INFECTION AND IMMUNITY, June 1990, p. 1986–1991
0019-9567/90/061986-06$02.00/0
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Vol. 58, No. 6

NOTES

Binding of Colloidal Gold-Labeled Salivary Proline-Rich Proteins to *Actinomyces viscosus* Type 1 Fimbriae

K.-P. LEUNG,[1,2] W. E. NESBITT,[1,2] W. FISCHLSCHWEIGER,[1] D. I. HAY,[3] AND W. B. CLARK[1,2]*

Department of Oral Biology[1] and Periodontal Disease Research Center,[2] College of Dentistry, University of Florida, Gainesville, Florida 32610, and Department of Biochemistry, Forsyth Dental Center, Boston, Massachusetts 02115[3]

Received 15 November 1989/Accepted 26 February 1990

Salivary proline-rich proteins (PRPs), which were purified from parotid saliva, were adsorbed onto 15-nm-diameter gold particles to visualize specific binding of the salivary molecules to *Actinomyces viscosus* type 1 fimbriae. Negatively stained preparations incubated with PRP-gold conjugates but not bovine serum albumin-gold complexes bound specifically to bacteria possessing type 1 fimbriae, *A. viscosus* T14V-J1 and 5519. Binding of the PRP-gold probes to strains deficient in type 1 fimbriae, i.e., strains 5951 (type 2 fimbriae only) and 147 (no fimbriae), was negligible.

Actinomyces viscosus T14V-J1 possesses two antigenic and functional types of fimbriae, types 1 and 2 (1, 2, 5, 6), which in recent years have been implicated in promoting the attachment and colonization of this organism to oral surfaces (1, 5). Using inhibition of adsorption to saliva-treated hydroxyapatite by anti-type 1 antibodies and their Fab fragments, Clark et al. (5) demonstrated that type 1 fimbriae mediate the attachment of strain T14V-J1 to salivary pellicles that were formed on surfaces similar to those of teeth. Type 2 fimbriae, on the other hand, have been shown to mediate adherence of *A. viscosus* to epithelial cells (1), certain oral streptococci (2), and neuraminidase-treated erythrocytes (6) by a different mechanism which involves lectin-carbohydrate-like interactions. Gibbons and Hay (9) and Gibbons et al. (10) have suggested that human parotid salivary proline-rich proteins (PRPs) present in the pellicle serve as receptors for the attachment of this bacterium, and recently, Clark et al. (4) have shown that immunoglobulin G and Fabs specific for type 1 fimbriae inhibited the specific adsorption of type 1-positive *A. viscosus* strains to hydroxyapatite treated with PRPs or proline-rich glycoprotein. While the role of salivary PRPs in mediating the attachment of *A. viscosus* appears clear, little is known about the interactions of these proteins with type 1 fimbriae. In this study, gold particles conjugated with purified PRPs (PRP-1 and its isoelectric focusing variant PIF-slow [PIF-s]) were used as probes to demonstrate ultrastructurally the specific binding of PRPs to *A. viscosus* type 1 fimbriae.

Several strains of *A. viscosus*, carrying different phenotypic expressions of fimbriae, were used in this study. These were strain T14V-J1 (both type 1 and 2 fimbriae) and its fimbria-deficient mutants 5519 (type 1 fimbriae only), 5951 (type 2 fimbriae only), and 147 (no fimbriae), which were kindly provided by J. O. Cisar (3), National Institute of Dental Research (Bethesda, Md.). All strains were maintained as multiple frozen stocks. For ultrastructural studies of binding, cells were grown aerobically overnight on Trypticase soy (BBL Microbiology Systems, Cockeysville, Md.) blood agar plates at 37°C.

Individual salivary PRPs (PRP-1 and PIF-s) were obtained and purified as described elsewhere (14). Their purity was assessed by polyacrylamide gel electrophoresis, high-performance liquid chromatography, and sequencing (9, 12). Colloidal gold sols, having an average diameter of 15 nm, were prepared as described by Goodman et al. (11). The pH of the suspension was adjusted to approximately 7.0 with 0.2 M potassium carbonate. Optimal amounts of proteins, i.e., PRP-1, PIF-s, or bovine serum albumin (BSA), for coating the gold particles were determined by the construction of a protein isotherm as described previously (8, 13). The optimal amount of each protein necessary to stabilize the gold particles was determined as the smallest amount of protein which prevented the gold sols from flocculation, i.e., a color change from red (adequate to excess protein) to blue (inadequate protein), after the addition of high concentrations of salt. For the preparation of gold complexes, a 10% excess of protein was added to the optimal protein amount. In our studies, 20 ml of 15-nm-diameter gold spheres was used to prepare gold conjugates routinely, and they could be stabilized with approximately 44 μg of PRP-1, 44 μg of PIF-s, or 1.1 mg of BSA. Furthermore, when the pH for establishing the PRP-gold conjugation was adjusted to a lower pH, i.e., 5.5, which was approximately 0.8 pH unit above the pI of PRPs, the conjugates appeared to be less stable (as tested by the addition of high concentrations of salt) and more aggregated.

For electron microscopy, the gold complexes were further processed as described by Slot and Geuze (16) with a slight modification. The pH of stabilized gold sols was adjusted to 9.0 with 0.2 M K_2CO_3, and 10% BSA in distilled H_2O (pH 9.0) was added to the suspension to a final concentration of 1% BSA. After incubation at room temperature with agitation for 5 min, the suspension of gold complexes was centrifuged at 49,000 × *g* for 45 min at 4°C in an ultracentrifuge (L5-65; Beckman Instruments, Inc., Fullerton, Calif.) by using an SW 40 Ti rotor. After the supernatant was discarded, the pellet was suspended in 20 mM Tris-saline (0.15 M NaCl) buffer (pH 8.2) containing 1% BSA to approximately 10% of the original volume. The suspension was then layered on an 8-cm-long, 10 to 30% (wt/wt) glycerol

* Corresponding author.

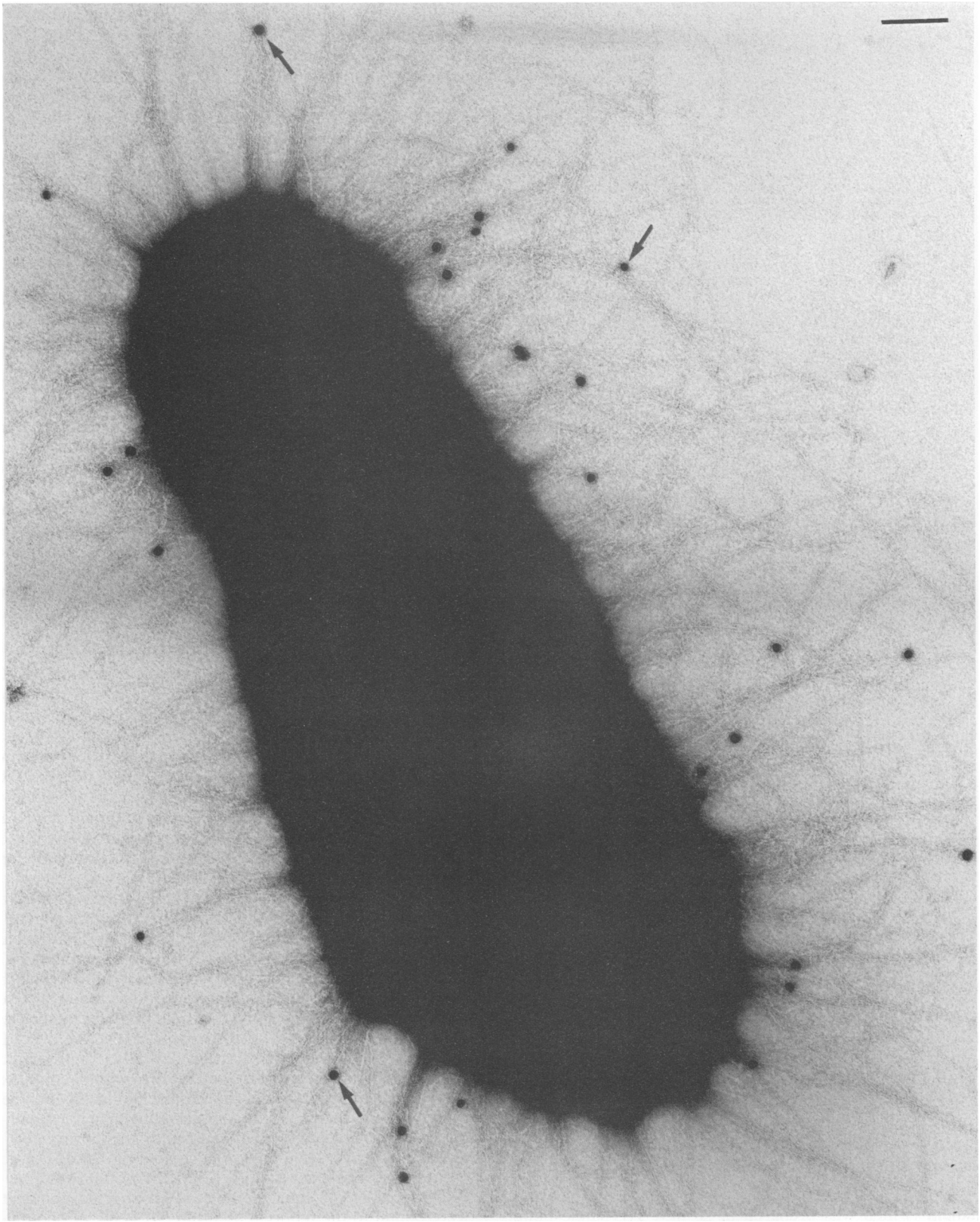

FIG. 1. Binding of salivary PRP-1-coated gold particles to *A. viscosus* T14V-J1. Many PRP-1-coated gold particles (arrows) bound to the fimbriae of T14V-J1 (type 1^+ 2^+). Bar, 0.1 μm.

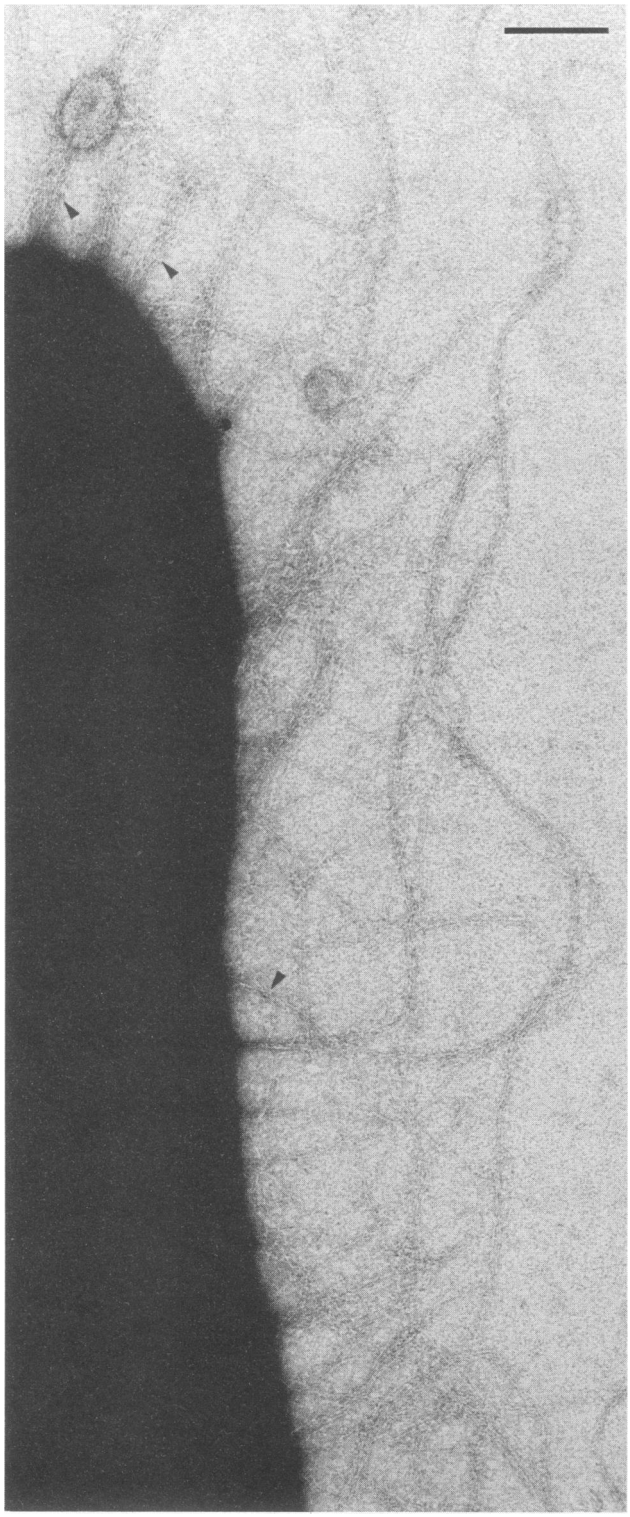

FIG. 2. Binding of BSA-coated gold particles to *A. viscosus* T14V-J1. Very few BSA-coated gold particles bound to the bacteria. Fimbriae are indicated by arrowheads. Bar, 0.1 μm.

gradient (which was prepared in 20 mM Tris-saline buffer containing 1% BSA). The gradient was centrifuged as described above for 30 min. After centrifugation, the middle to lower one-third of the gradient, which contained stabilized and uniformly dispersed gold spheres, was collected and dialyzed against the same buffer to remove the glycerol. The resulting gold complexes were then passed through a membrane filter (pore size, 0.45 μm) and stored in sterile vials at 4°C. The probes that were prepared appeared to be stable for at least 3 months. Cells of *A. viscosus* and its fimbria-deficient mutants, from late-exponential or stationary-phase cultures, were obtained from Trypticase soy blood agar plates and suspended gently in 20 mM Tris-saline buffer. They were then negatively stained as described by Robinson et al. (15), with modification. Briefly, the bacterial suspension was deposited on carbon-stabilized, Formvar-coated nickel grids (200 or 300 mesh; Ted Pella Inc., Redding, Calif.). After 1 min, excess fluid was removed with filter paper strips, and the grids were transferred to drops of 20 mM Tris-saline buffer containing 1% BSA for blocking of nonspecific binding sites. All incubations were performed at room temperature. After blocking was done twice for 15 min each time, grids adsorbed with T14V-J1 cells or their fimbria-deficient mutants were floated for 60 min on drops of one of the following conjugates: (i) PRP-1–gold, (ii) PIF-s–gold, or (iii) BSA-gold. The grids were then washed in distilled H_2O four times and stained with 1% aqueous uranyl acetate for 30 s. All experiments were repeated at least twice. Two concentrations (as determined by the optical density at a wavelength of 520 nm) of PRP-gold conjugates were used in the labeling study. The negatively stained specimens were examined in an electron microscope (EM 10A; Zeiss) at an accelerating voltage of 80 kV. The binding of various gold conjugates to *A. viscosus* strains was analyzed by the Kruskal-Wallis one-way analysis of variance test and further compared by the Wilcoxon nonparametric test.

Negative staining of the samples showed that PRP-1–gold complexes (Fig. 1) bound to the fimbriae of strain T14V-J1, whereas many fewer BSA-gold particles bound to this organism (Fig. 2). Incubation of the gold probes with fimbria-deficient mutants of strain T14V-J1 revealed that PRP-1–gold (Fig. 3A) or PIF-s–gold (Fig. 3B) conjugates bound to strains 5519 (type 1 fimbriae only). The same gold probes, PRP-1–gold or PIF-s–gold conjugates, failed to bind to 5951 (type 2 fimbriae only; Fig. 4A) or 147 (no fimbriae; Fig. 4B), respectively. Labeling of fimbriae appeared to be scattered, with many gold conjugates localized distally and some localized more proximally (Fig. 1 and 3).

In order to gain semiquantitative information related to the binding, gold conjugates (PRP-1–gold, PIF-s–gold, or BSA-gold) which bound to strain T14V-J1 and its fimbria-deficient mutants were counted, and the results are summarized in Table 1. Thirty single cells that were clearly visible and that exhibited little or no clumping were selected from each sample, and an average number of gold particles per cell was determined. Virtually no gold particles (PRP-1–gold or PIF-s–gold) were associated with strain 147 cells. Approximately four PRP-1–gold conjugates or six PIF-s–gold complexes were found in association with strain 5951 cells. For cells carrying type 1 fimbriae, a significantly higher number of fimbriae associated with gold conjugates was observed ($P < 0.0001$). An average of 20 PRP-1–gold or 24 PIF-s–gold particles were associated with T14V-J1 fimbriae, whereas 20 PRP-1–gold or an equal number of PIF-s–gold conjugates were bound to the fimbriae of strain 5519 cells. In comparison, binding of BSA-gold complexes to strains T14V-J1 (two particles per cell; $P < 0.0001$) and 5519 (one particle per cell; data not shown in Table 1) was negligible. The data collec-

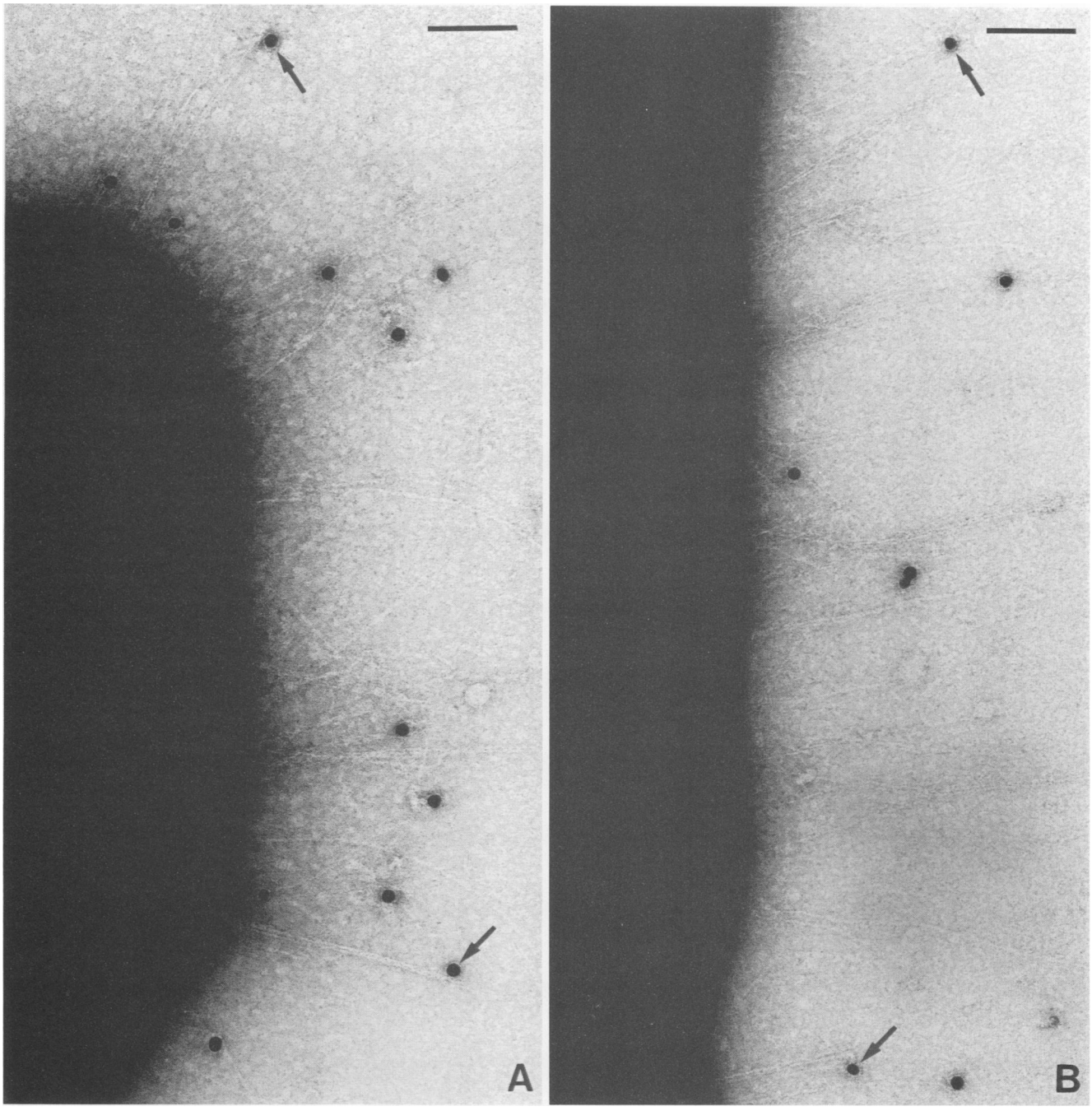

FIG. 3. PRP-1–gold (A) or PIF-s–gold (B) conjugates (arrows) bound to the fimbriae of strain 5519. Bars, 0.1 μm.

tively suggested that PRP-gold complexes bind specifically to *A. viscosus* type 1 fimbriae.

Gibbons and Hay (9) and Gibbons et al. (10) have demonstrated that salivary PRPs in the adsorbed state on experimental pellicles may serve as receptors for the attachment of *A. viscosus* in vitro. However, PRPs, when present in soluble forms, did not interact with *A. viscosus* cells. Gibbons and Hay (9) suggested that functional molecular segments of PRPs which react with *A. viscosus* adhesins become exposed when the proteins adsorb to the surface. In this study with colloidal gold labeling, we extended previous observations by ultrastructural demonstration of the specific binding of purified PRPs to *A. viscosus* type 1 fimbriae. The working assumption was that individual purified PRPs (PRP-1, PIF-s) would be adsorbed on gold surfaces and become functional, similar to PRPs that are adsorbed on experimental pellicles. Interactions of PRP-1-, PIF-s-, or BSA-gold with *A. viscosus* T14V-J1 demonstrated the specific binding of the PRP-gold to the fimbriae of the organism. PIF-s (a positional isomer of PRP-1), which has the reverse arrangement of amino acid residues of PRP-1 at positions 4 and 50, respectively (12), bound similarly to *A. viscosus* fimbriae. The use of the mutants confirmed the correlation between the presence of type 1 fimbriae and binding of the PRP-gold probes.

One of the intriguing observations in this study was the

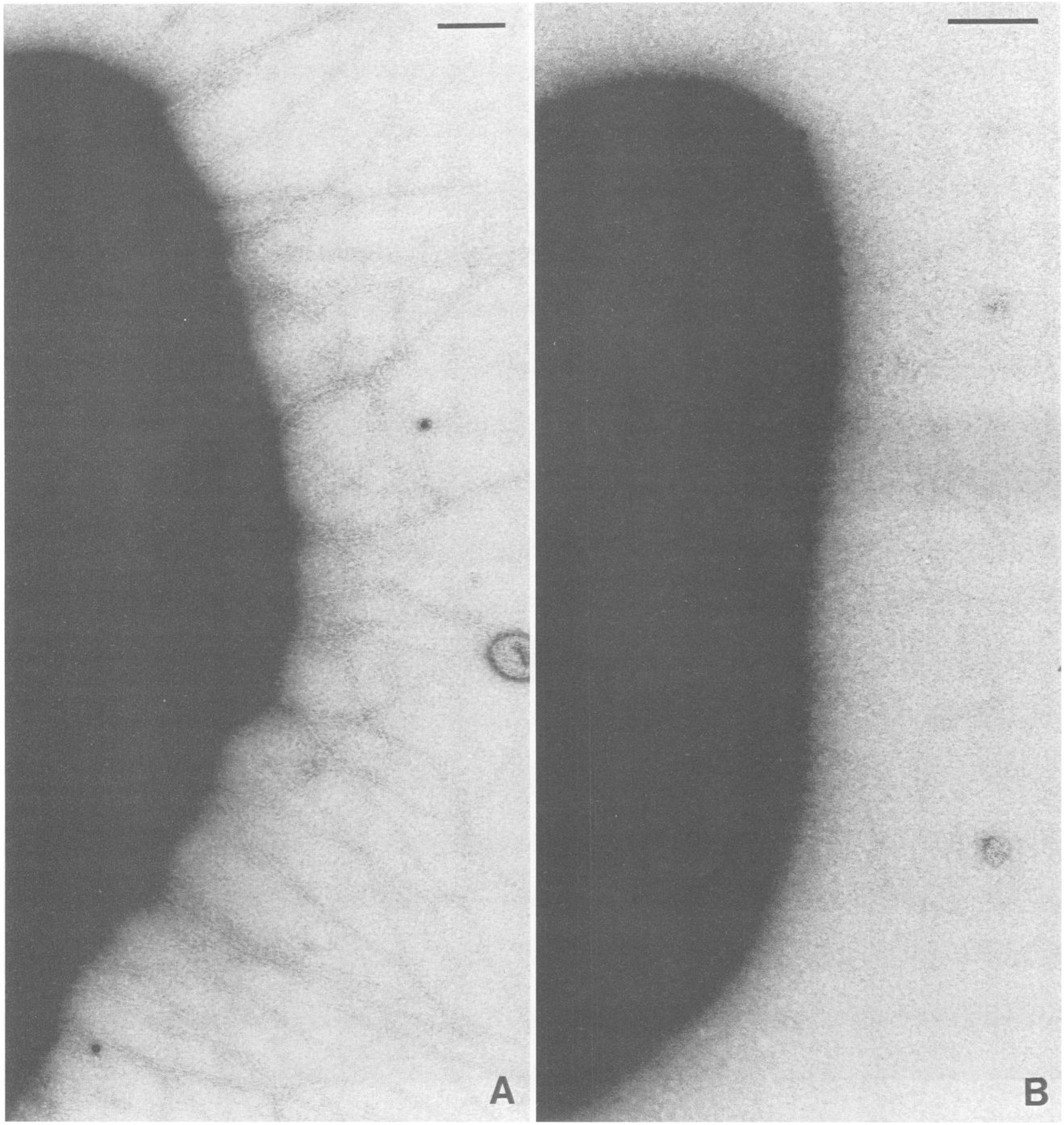

FIG. 4. *A. viscosus* 5951 (type 2 fimbriae only) (A) or 147 (no fimbriae) (B) was reacted with PRP-1–gold or PIF-s–gold conjugates, respectively. Very few conjugates bound to the fimbriae or the cell surface of the bacteria. Bars, 0.1 μm.

distinct binding pattern of PRP-gold conjugates on strain T14V-J1 and 5519 fimbriae. Unlike immunogold labeling with anti-type 1 (*A. viscosus*) antibodies, which showed that the labels were distributed extensively along the fimbriae (7), most PRP-gold complexes appeared to exhibit a single site of binding to the fimbriae. The difference in the binding pattern observed may be due to the fact that the anti-type 1 antibodies used were directed principally against the structural subunits of *A. viscosus* fimbriae (J. O. Cisar, E. L. Barsumian, R. P. Siraganian, W. B. Clark, M. K. Yeung, S. D. Hsu, S. H. Curl, A. E. Vatter, and A. L. Sandberg, submitted for publication), whereas most PRP-gold conjugates that were used presumably reacted with PRP-reactive sites on the fimbriae. Recently accumulated evidence suggests that the major component of *A. viscosus* type 1 fimbriae is the structural subunit, whereas the fimbrial adhesin(s) for PRPs may represent the minor component (Cisar et al., submitted). Furthermore, one question deserving consideration is whether the labeling shown in this study represents the true distribution of PRP-binding sites. Be-

TABLE 1. Binding of various gold conjugates to *A. viscosus* strains

Gold conjugate	Bacterial strain (fimbrial phenotype)	No. of bacteria counted	Average no. of gold conjugates/ bacterium[a]
PRP-1	T14V-J1 (1^+ 2^+)	52	20 ± 9^b
PIF-s	T14V-J1 (1^+ 2^+)	50	24 ± 11^b
PRP-1	5519 (1^+ 2^-)	50	20 ± 6^b
PIF-s	5519 (1^+ 2^-)	50	20 ± 11^b
PRP-1	5951 (1^- 2^+)	50	4 ± 3^c
PIF-s	5951 (1^- 2^+)	30	6 ± 5^d
PRP-1	147 (1^- 2^-)	35	0.08 ± 0.4^e
PIF-s	147 (1^- 2^-)	45	1 ± 1^e
BSA	T14V-J1 (1^+ 2^+)	50	2 ± 2^f

[a] Values are means ± standard deviations. Bacterial cells were analyzed by the Kruskal-Wallis one-way analysis of variance test and were further compared by the Wilcoxon nonparametric analysis. Mean values with different letters were significantly different ($P < 0.005$).

cause of the limitation of the ultrastructural approach, it is not certain that the optimal binding of PRP-gold conjugates to *A. viscosus* type 1 fimbriae was achieved. However, two different concentrations of gold probes were used for labeling the adhesins of *A. viscosus* fimbriae. Little difference in the binding pattern was observed when the two concentrations of probes interacted with strain T14V-J1. *A. viscosus* cells incubated with a higher concentration of PRP-gold conjugates are shown in Fig. 1 to 4. In summary, the results of this study extend the understanding of interactions of PRPs with *A. viscosus* type 1 fimbriae, and this study is the first of its kind to locate ultrastructurally the fimbria-associated PRP-reactive sites. The binding pattern reported here suggests that the location for salivary PRPs on type 1 fimbriae is unique or that the adhesin(s) may represent the minor component(s) of the fimbriae.

We thank A. Bennick for helpful discussions, Phil Padgett for statistical analysis, and Jenny van Mill for assistance in typing and editing the manuscript. We also acknowledge the support provided by the Electron Microscopy Facility of the College of Dentistry.

This research was supported by Public Health Service grants DE 05429 and DE 07117 (to W.B.C.), DE 7009, and DE 8601 (to D.I.H.) from the National Institute of Dental Research.

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